Distribution of *Cytophagales* in a tidal pond of Great Sippewissett Salt Marsh.

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Microbial Diversity Course, 1999.

Abstract

The distribution of *Cytophagales* in a tidal pond containing berries was examined. DNA was extracted from berries, pond mud and water. Gliding bacteria belonging to the flavobacterium-cytophaga group were isolated from berry rinse water and pond mud. All isolates grew on low nutrient seawater agar plates with tryptone-yeast extract supplied as carbon source16S rDNA in the extracts were amplified using primers specific to the flavobacterium-cytophaga branch of the *Cytophagales*.

Introduction

The *Cytophagales* division is one of the major lineages of bacteria (Hugenholtz *et al.* 1998). Within this division members of the genera *Cytophaga*, *Flavobacterium*, and *Flexibacter* are frequently isolated from natural and modified ecosystems including soil, fresh and marine water and sewage treatment plants. They are gliding, rod-shaped to filamentous bacteria. Most are obligately aerobic, but some are fermentative. Because of their gliding motility their colonies are thin and spreading, almost always with a yellow, orange or red pigmentation. The genera *Cytophaga* and *Flexibacter* are best known for their ability to degrade biomacromolecules such as starch, cellulose, gelatin, chitin, agar and xylan.

Cloning and sequencing of 16S rDNA extracted from berries from a tidal pond of the Great Sippewissett Salt Marsh (Seitz *et at.* 1993) revealed the presence of bacteria belonging to the *Cytophagales* (Banin 1997). The clones obtained were most similar to cultured strains of *Cytophaga fermentans*, *C. salmonicolor* and *C. agarovorans*, all of which are marine facultatively anaerobic species. To investigate further the distribution of *Cytophagales*, in particular gliding bacteria, in a tidal pond, a combination of phylogenetic and isolation techniques were applied to mud, water, mussels and berries.

Materials and Methods

Direct isolation of gliding bacteria from environmental samples

Bacteria capable of degrading biomacromolecules were isolated directly from berries or tidal pond mud. Dilutions of homogenised washed berries, berry rinse water or tidal pond mud in sterile seawater were spread onto low nutrient seawater agar supplemented with either tryptone-yeast extract, cellulose, or xylan as carbon source, or chitin as carbon and nitrogen source at a final concentration of 0.2-0.5% (w/v). The seawater medium contained: 0.5 g/l K_2HPO_4, 1 g/l NH_4Cl, 16 g/l agar (Difco), 300 ml distilled water and 700 ml seawater. For chitin containing plates NH_4Cl was omitted. Plates were incubated at room temperature under aerobic and anaerobic conditions. Thin spreading colonies were removed from isolation plates and examined using phase microscopy for the presence of gliding, rod-shaped to filamentous bacteria. The gliding bacteria were purified by either streaking inocula from the edge of the colonies onto seawater medium with tryptone-yeast extract as carbon source or by diluting gliding colonies in sterile seawater and then plating onto marine agar (Difco).

Isolates were screened for the production of flexirubin by flooding colonies with 20% (w/v) potassium hydroxide. Their ability to grow on tryptone-yeast extract, cellulose, chitin, xylan, and laminarin was determined by comparison with substrate-free control plates. Growth under anaerobic conditions was determined on seawater plates containing tryptone-yeast extract.

16S rDNA sequence analysis of pure cultures

DNA was extracted from cultures using the Prepman™ method from the MicroSeq™ 500 16SrDNA Bacterial Sequencing kit (Applied Biosystems). Purified DNA from strain 35 was amplified using the universal eubacterial primers 8F and 1492R whereas the forward primer 319aF specific for the flavobacterium-cytophaga branch of the *Cytophagales* and the eubacterial reverse primer 1492R were used for strains 13, 17 and 30. The PCR amplification cycling parameters were as follows: (94°C, 12 min) x 1, (94°C, 1.5 min; 55°C, 1 min; 72°C, 1 min) x 30, (72°C, 12 min) x 1. PCR products were confirmed by gel electrophoresis using the 1kb DNA ladder size marker. Sequence data was analysed using programs from ARB and a phylogenetic tree was constructed using neighbour joining.

Enrichment cultures for fermentative gliding bacteria

Cultures to enrich for fermentative gliding bacteria were established in seawater medium supplemented with 0.2-0.5% (w/v) chitin, cellulose or xylan and pond mud served as source of inocula. Tubes were filled to exclude oxygen. All tubes were incubated with shaking at room temperature.

Whole cell fluoresecent in situ hybridization (FISH).

An rRNA-targeted oligonucleotide probe Flavo labelled with rhodamine which is specific for the *Cytophagales* was used on isolates and to determine whether enrichment cultures may a source of gliding bacteria. The samples were prepared for FISH analysis and counterstained with DAPI using the methods described in the Microbial Diversity course notes.

Extraction and analysis of DNA from environmental samples

DNA was extracted from tidal pond mud, water, and berries and the contents of a mussel gut using the MoBio Soil kit. DNA was amplified using the forward primer 319aF and the reverse primer 1492R. PCR products were cloned into *Escherichia coli* using the TOPO TA™ cloning kit (Invitrogen). Clones containing inserts of the expected size were sent for sequence analysis.

Results

Four isolates of gliding bacteria were obtained, two from berry rinse water and two from pond mud (Figure 2; Table 1). Attempts to isolate gliding bacteria from mussel gut contents or pond water were unsuccessful. All of the isolates grew on tryptone-yeast extract, and all isolates except 35, utilised the carbohydrates cellulose and chitin for growth. None of the isolates produced flexirubin pigments. 16S rDNA sequence analysis confirmed that isolates 13, 30 and 35 belonged to the *Cytophagales*. Sequence information from isolate 17 was of poor quality, however the bacterium did

hybridise to the Flav probe when used for FISH analysis indicating that it also belonged to the *Cytophagales* (results not shown).

FISH analysis suggests that fermentative *Cytophagales* can be enriched from tidal pond mud when cellulose, chitin or xylan is supplied as carbon source. Approximately 30-50% of the cells in the enrichment culture growing with xylan as carbon source stained with DAPI, hybridised to the Flav probe (Figure 2 a & b). Similar observations were made for enrichments with cellulose or chitin (results not shown). Most of the cells appeared to be small, some were rod-shaped but few filaments were observed.

DNA extracted and amplified from the tidal pond mud, water and berries using the flavobacteria-cytophaga specific forward primer, indicates that these samples contain bacteria belonging to the *Cytophagales*. Sequence was obtained for four clones and their similarity to known organisms determined (Table 2). Only one of the clones mud 29 belonged to the *Cytophagales*. DNA was not successfully extracted from mussel gut contents.

A 16S rDNA phylogenetic tree showing the positions of isolates 13, 30 and 35 and mud clone 29 was produced (Figure 3). The isolates grouped with *Cytophagales* of marine origin. Isolates 13 and 35 grouped with the fish pathogen *Flexibacter maritimus* and the psychrophile *Polaribacter filamentus,* whereas isolate 30 grouped with *C. latercula* from a marine aquarium. Mud clone 29 clustered with *Sphingobacterium* spp.

Discussion

Studies of *in situ* bacterial diversity reveal the prevalance of *Cytophagales* in the environment (Manz *et al.* 1996; Hugenholtz *et al.*, 1998). In this investigation of a tidal pond of the Great Sippewissett Salt Marsh, *Cytophagales* were isolated from berry rinse water and mud, enriched from the mud when biomacromolecules were used as carbon source, and a clone obtained. Amongst the population of bacteria that grew on low nutrient-seawater plates, colonies indicative of gliding bacteria were not common, despite the high numbers of *Cytophagales* observed in enrichment cultures (Figure 2). These results indicate that pre-enrichment of samples may be a worthwhile strategy for obtaining *Cytophagales* from the environment. Alternatively selection of colonies that exhibit gliding behaviour may exclude the isolation of many of the *Cytophagales* which are present in nature. Furthermore, filamentous bacteria were rarely observed in enrichment cultures with high numbers of bacteria that hybridised to the Flav probe.

Molecular probes provide a powerful tool for the study of bacteria in nature however the specificity of the probes used is of extreme importance. The 319a F primer used to amplify DNA extracted from environmental samples hybridises to members of the *Cytophagales* and a few gram-positive bacteria (Bruns & Berthe-Corti, 1998). Further evidence for the lack of specificity of these primers can be inferred from this study. Of the four clones obtained using the 319a F primer and subsequently sequenced only

one was a *Cytophagales*. The lack of sequence information obtained however (in some cases less than 200 base pairs) must be taken into consideration.

In summary, the evidence indicates that *Cytophagales* are distributed in tidal ponds of the Great Sippewissett Salt Marsh. Bacteria isolated thus far are not the same as those cloned from this environment. The use of pre-erichment techniques may increase the opportunity to isolate further strains such as *C. fermentans*, which are more similar to those cloned previously from this environment.

Acknowledgements

I thank my group members for their help with this project, and Scott Dawson and Dr Tom Schmidt for increasing my understanding of, and confidence to use molecular tools

References

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Bruns, A.; Berthe-Corti L. 1998. *In situ* detection of bacteria in continuous-flow cultures of seawter sediment suspensions with fluorescently labelled rRNA-directed oligonucleotide probes. Microbiology 144: 2783-2790.

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Table 1: Gliding bacteria isolated from a tidal pond

Isolate #	13	17	30	35
Source	berries	Berries	mud	mud
Isolated on:	xylan	Laminarin	cellulose	xylan
Colony pigmentation	yellow	Yellow	yellow-orange	yellow
Facultative aerobes	-[a]	-	+[b]	-
Growth on:				
tryptone-yeast extract	+	+	+	+
Cellulose	+	+	+	-
Chitin	+	+	+	-
Laminarin	+	+	-	-
Xylan	+	ND	+	+
16Sr DNA sequence similarity (%)	*Flavobacterium* (96%)	ND	*Cytophaga* (95%)	*Flexibacter* (91%)

[a] means no growth
[b] means growth
[c] means not done.

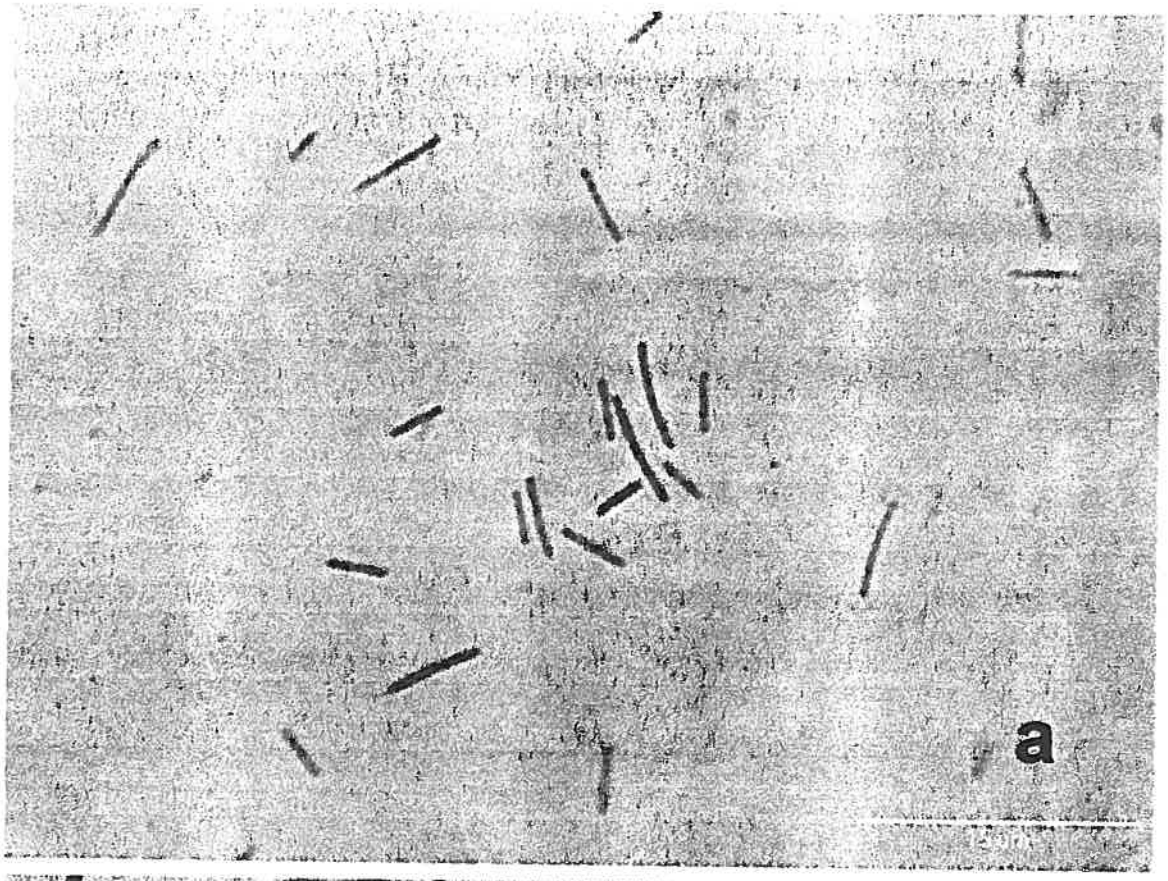
Table 2: Sequence similarity of clones obtained from the tidal pond with known organisms

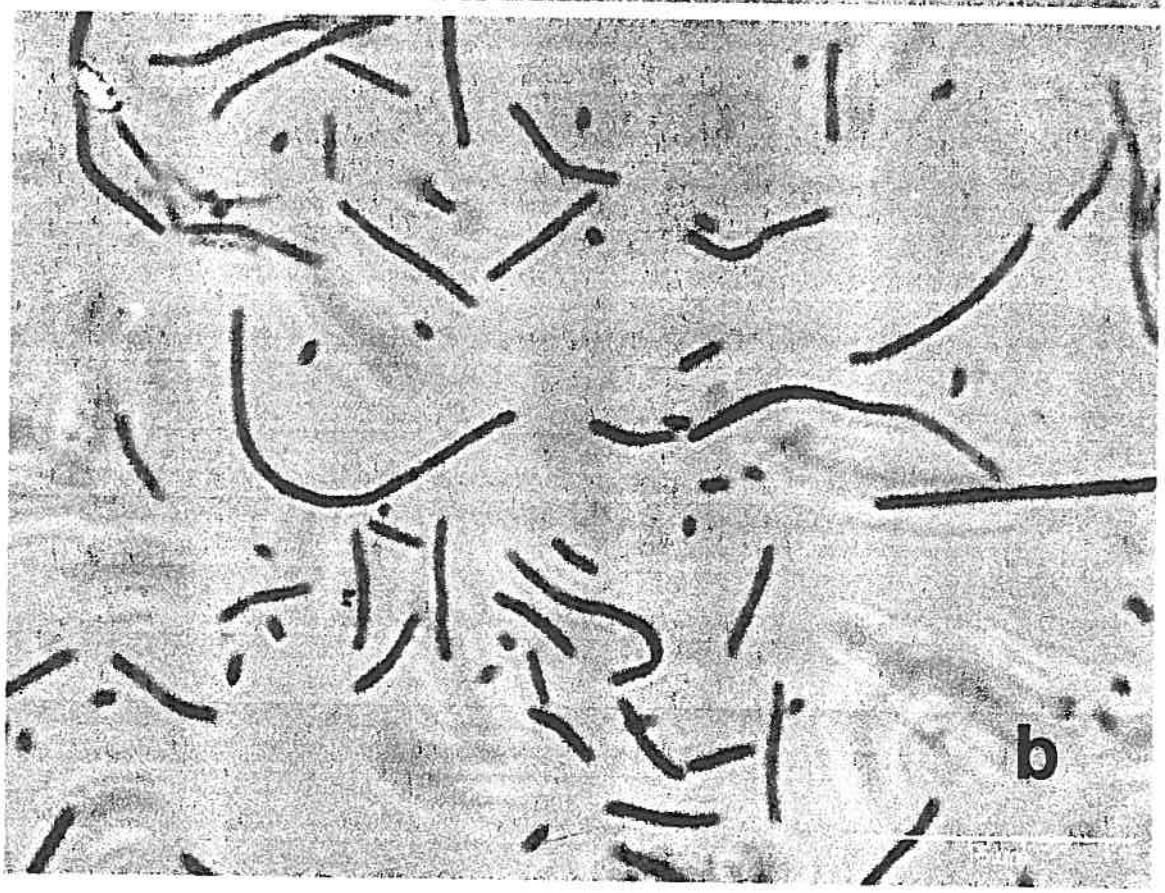
Clone #	**Source**	**Closest organism match on BLAST search (% similarity)**
26	Mud	Actinomycete (97%)
27	Mud	Uncultured eubacterium (98%)
29	Mud	*Cytophagales* (93%)
33	Water	Chloroplast (98%)

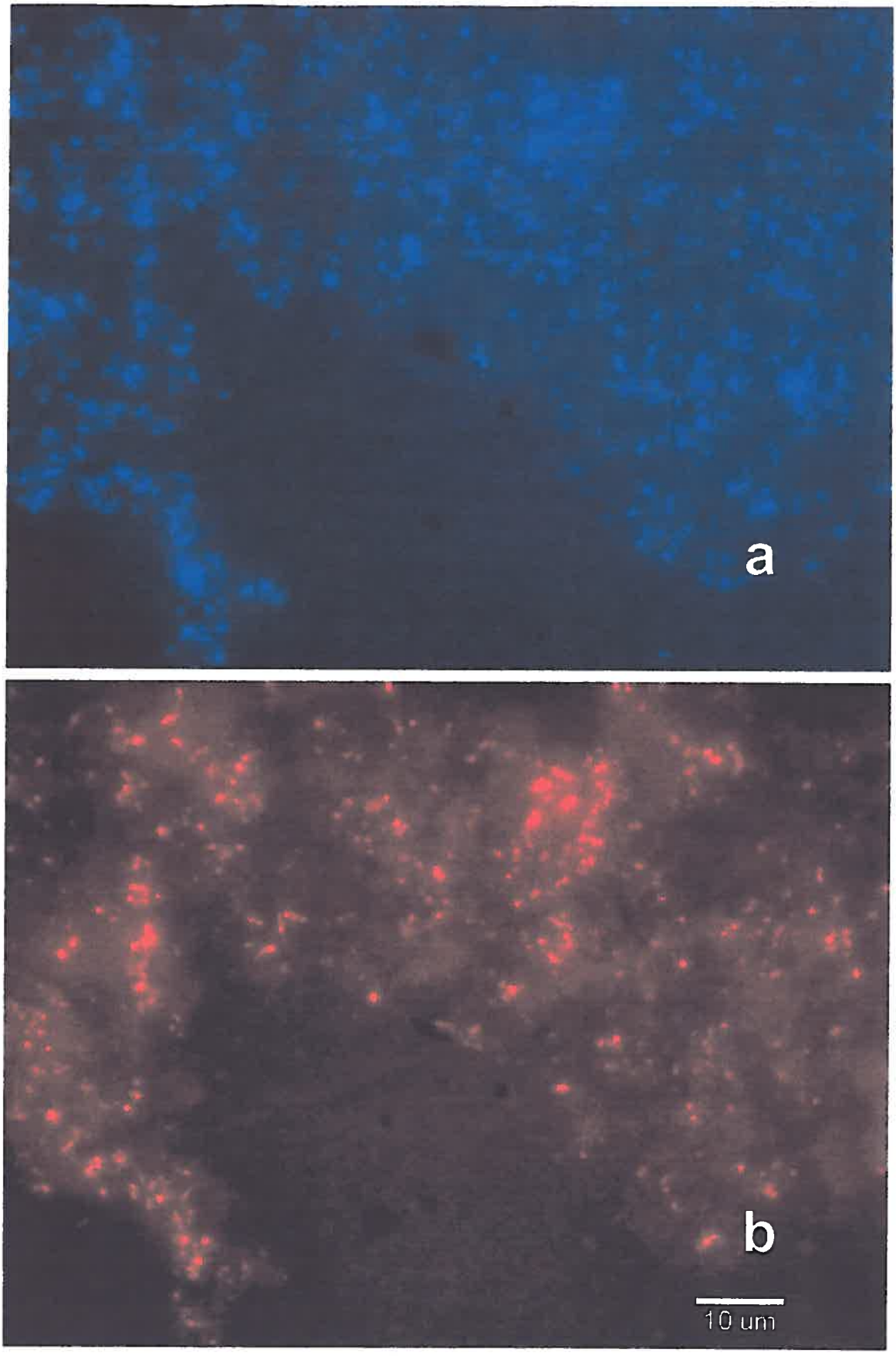
Figure 1. *Cytophagales* isolates (a) 13 and (b) 17.

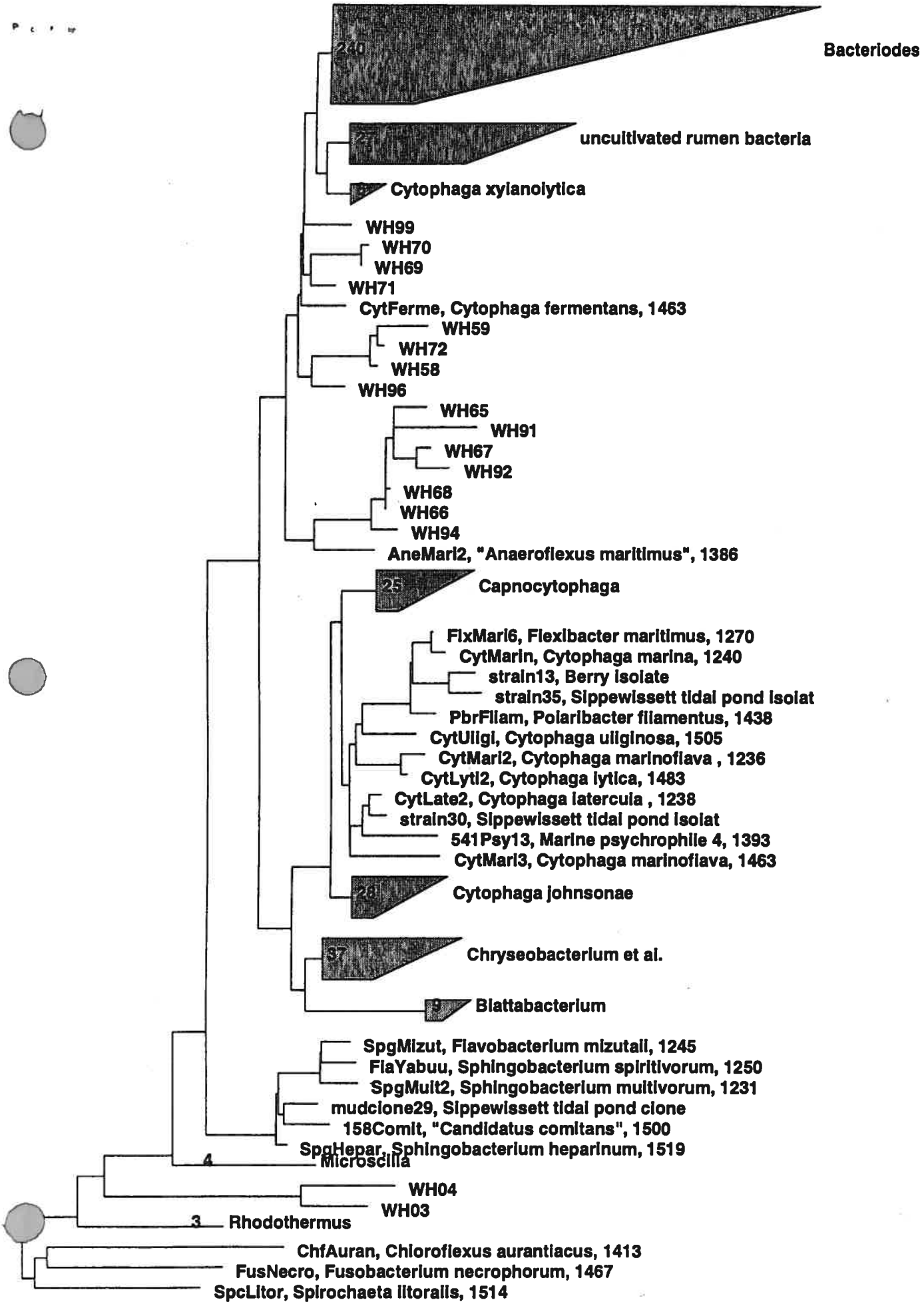
Figure 2. FISH of an enrichment culture from tidal pond mud grown on xylan and stained with DAPI (a) and the probe flav that is specific for *Cytophagales*.

Figure 3. Phylogenetic tree of selected *Cytophagales* showing the relationship of the isolates (13, 30 and 35) and mud clone 29 to known bacteria .


a


b


a
b
10 um


240
Bacteriodes
uncultivated rumen bacteria
Cytophaga xylanolytica
WH99
WH70
WH69
WH71
CytFerme, Cytophaga fermentans, 1463
WH59
WH72
WH58
WH96
WH65
WH91
WH67
WH92
WH68
WH66
WH94
AneMari2, "Anaeroflexus maritimus", 1386
25
Capnocytophaga
FlxMari6, Flexibacter maritimus, 1270
CytMarin, Cytophaga marina, 1240
strain13, Berry isolate
strain35, Sippewissett tidal pond isolat
PbrFilam, Polaribacter filamentus, 1438
CytUligi, Cytophaga uliginosa, 1505
CytMari2, Cytophaga marinoflava , 1236
CytLyti2, Cytophaga lytica, 1483
CytLate2, Cytophaga latercula , 1238
strain30, Sippewissett tidal pond isolat
541Psy13, Marine psychrophile 4, 1393
CytMari3, Cytophaga marinoflava, 1463
28
Cytophaga johnsonae
37
Chryseobacterium et al.
9
Blattabacterium
SpgMizut, Flavobacterium mizutaii, 1245
FlaYabuu, Sphingobacterium spiritivorum, 1250
SpgMult2, Sphingobacterium multivorum, 1231
mudclone29, Sippewissett tidal pond clone
158Comit, "Candidatus comitans", 1500
SpgHepar, Sphingobacterium heparinum, 1519
4
Microscilla
WH04
WH03
3
Rhodothermus
ChfAuran, Chloroflexus aurantiacus, 1413
FusNecro, Fusobacterium necrophorum, 1467
SpcLitor, Spirochaeta litoralis, 1514
0.10